American Fern Journal 104(2):58–66 (2014)

Effects of Seasonal Changes on Pigment Composition of *Azolla filiculoides* Lam.

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ABSTRACT.—*Azolla* is a floating fern, which contains the nitrogen-fixing cyanobacterium *Anabaena azollae* in the dorsal lobe cavity of the leaves. This study investigated the effects of seasonal changes on the pigment composition of *Azolla filiculoides* in a small pond located in Istanbul, Turkey. Sampling was conducted in March, April, May, October, November, December 2007. The average total chlorophyll and carotenoid content of the leaves was almost a half fold lower in March-April-May than in October-November-December. For late winter–spring, while the average chlorophyll a/b ratio of the leaves was 2.6, for autumn - early winter it was 5.5. In March, leaf dimensions reduced, whereas the anthocyanin accumulation in the leaves increased. In May, together with the increasing temperature values, while the leaf dimensions and biomass increased, the leaves returned to green. From October to November, both leaf dimensions and amount of anthocyanin in the leaves reduced. Results showed that the probable growth season of the *A. filiculoides* began between April and May.

KEY WORDS.—*Azolla*, chlorophyll, anthocyanin, carotenoids, seasonal variations

Azolla is a genus of floating ferns that contains a permanent endosymbiotic community of a heterosist-forming, filamentous, nitrogen-fixing cyanobacteria *Anabaena azollae* Strasb. living in the dorsal lobe cavity of the leaves (Peters and Mayne, 1974). *Azolla* has a worldwide distribution, ranging from temperate to tropical climates. Seven species of *Azolla* in two taxonomic sections are known: the section *Euazolla* includes the species *A. filiculoides* Lam., *A. rubra* R. Br., *A. mexicana* C. Presl, *A. caroliniana* Willd and *A. microphylla* Kaulf., while the section *Rhizosperma* includes the species *A. pinnata* R. Br. and *A. nilotica* Mett. (Van Hove and Lejeune, 2002).

Plant phenology is affected by internal factors or by environmental factors (Lee *et al.*, 2009). It is well known that seasonal changes affect plant growth and physiology. Ferns are sensitive components of ecosystems and display a seasonal acclimation of some physiological changes to changing environmental conditions (Volkova *et al.*, 2011). Stress conditions may lead to different physiological responses during a plant's different growth stages (Gratani *et al.*, 1998). Green *Azolla* leaves turn red under high light intensity, low temperature, and adverse environmental conditions (Pabby *et al.*, 2004). Photosynthetic pigments (chlorophylls and carotenoids), are responsible for the absorption of light (Lichtenthaler, 2009). The changes of the photosynthetic pigments and the photosynthetic performance affected by seasonal variations has been observed in many fern species (Volkova *et al.*, 2011; Paoli and Landi, 2013). Anthocyanins are partly responsible for the colors of plant tissues. Anthocyanins are produced in the cytoplasm, and then transported into the vacuole (Shirley, 1996). The synthesis of anthocyanins is induced by UV-B (Warren *et al.*, 2003), nutrient deficiency (Pinto *et al.*, 1999), low temperatures (Rabino and Mancinelli, 1986), water deficit (Nogués *et al.*, 1998), and heavy metal stress (Ling-Peng *et al.*, 2006). The content of the most common plant pigments—chlorophylls, carotenoids, and anthocyanins—as well as their relative proportion determines both the color and appearance of plants (Abbott, 1999).

In Turkey *Azolla filiculoides* is found naturally only in the Thrace region (Unal and Uzen, 1996) and has been grown in Istanbul University Alfred Heilbronn Botany Garden for many years. The purpose of this study was to monitor and investigate the effects of seasonal changes on *Azolla filiculoides* Lam. For this reason, changes in the pigment content of *A. filiculoides* were investigated in 2007.

Materials and Methods

Plant material and sampling.—The *Azolla filiculoides* that is grown in Istanbul University Alfred Heilbronn Botany Garden thrives in a small pond (N 41°01′06″, E 28°57′51″) with *Lemna* spp. Pigment analysis of *A. filiculoides* is based particularly on the months of previous years where there were significant temperature increases and decreases. For this reason *A. filiculoides* was collected from the botany garden in March, April, May, October, November and December of 2007. Five replicates of plants were collected from the different parts of the pond for each month sampled. After washing three times with distilled water, plant fresh weight was measured and pigment contents of the leaves determined.

Seasonal temperature.—To investigate the seasonal temperature changes in Istanbul, maximum, minimum, and mean daily temperature, data provided by the National Meteorology Service from the Florya meteorological station in Turkey was analyzed.

Chlorophyll and carotenoid levels.—To determine the chlorophyll and carotenoid contents of the leaves in March, April and May, 2–4 *A. filiculoides* plants totaling 700 mg leaves were used, and for October, November and December 4–6 plants totaling 500 mg leaves were used. Leaves were extracted in 80% acetone and the samples were centrifuged (Heraeus Labofuge 400 R) at 3000 g (4°C) for 15 minutes. The pigment contents (chlorophyll a and b, total chlorophyll, and carotenoid) were measured (Shimadzu 1601 UV-Visible Spectrophotometer) and determined in $\mu g\ g^{-1}$ fresh weight (Lichtenthaler and Welburn, 1983).

Anthocyanin determination.—The Mancinelli (1990) method was employed for the determination of the anthocyanin content in the leaves. According to this method, plant samples of 500 mg of fresh weight were extracted in 10 ml methanol-HCl (1% HCl, v/v) and kept under refrigeration at 3–5° C for 2 days with occasional shakings. Then, the extract was filtered and the anthocyanin content in the filtrate was measured (Shimadzu 1601 UV-Visible Spectrophotometer). The anthocyanin content was expressed as $\mu g\ g^{-1}$ fresh weight.

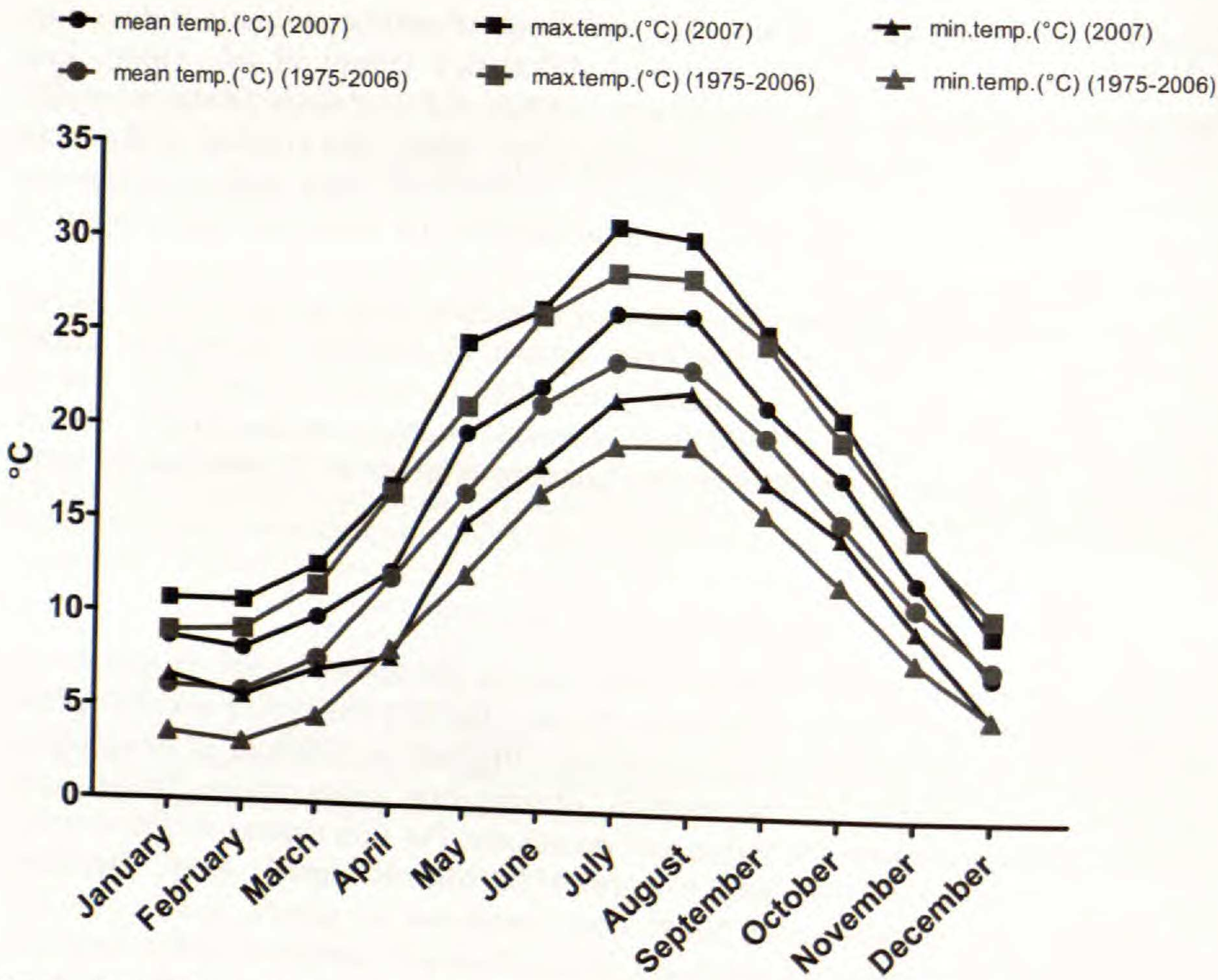

FIG. 1. Comparison of the maximum, minimum, and mean temperatures of Istanbul the years 2007 and 1975 to 2006 (data was provided by the National Meteorology Service, from Florya meteorological station in Turkey).

Statistical analysis.—Means of the five replicates of the chlorophyll, carotenoid, and anthocyanin content data were calculated. Statistical analysis was performed using GraphPad Prism version 5.2 for windows (GraphPad Software, San Diego, CA). The significance of differences between the mean values of samples were statistically evaluated by one way ANOVA followed *post hoc* analysis with the Tukey's Multiple Comparison test, $p<0.05$.

RESULTS

The mean and maximum temperatures of 2007 were high compared to the mean and maximum temperatures of those between 1975–2006; not including December. On the other hand the minimum temperatures in April and December of 2007 were lower than those of previous years (Fig. 1).

In Fig. 2, plant color, size and morphology during March-April-May and October-November-December 2007 are shown. In March (under the lowest spring temperature) the plants were small, a dark red color, and the growth of plants was very slow. At the beginning of April, the plants' color changed from red to green, plant growth increased, and vegetative multiplication accelerated.

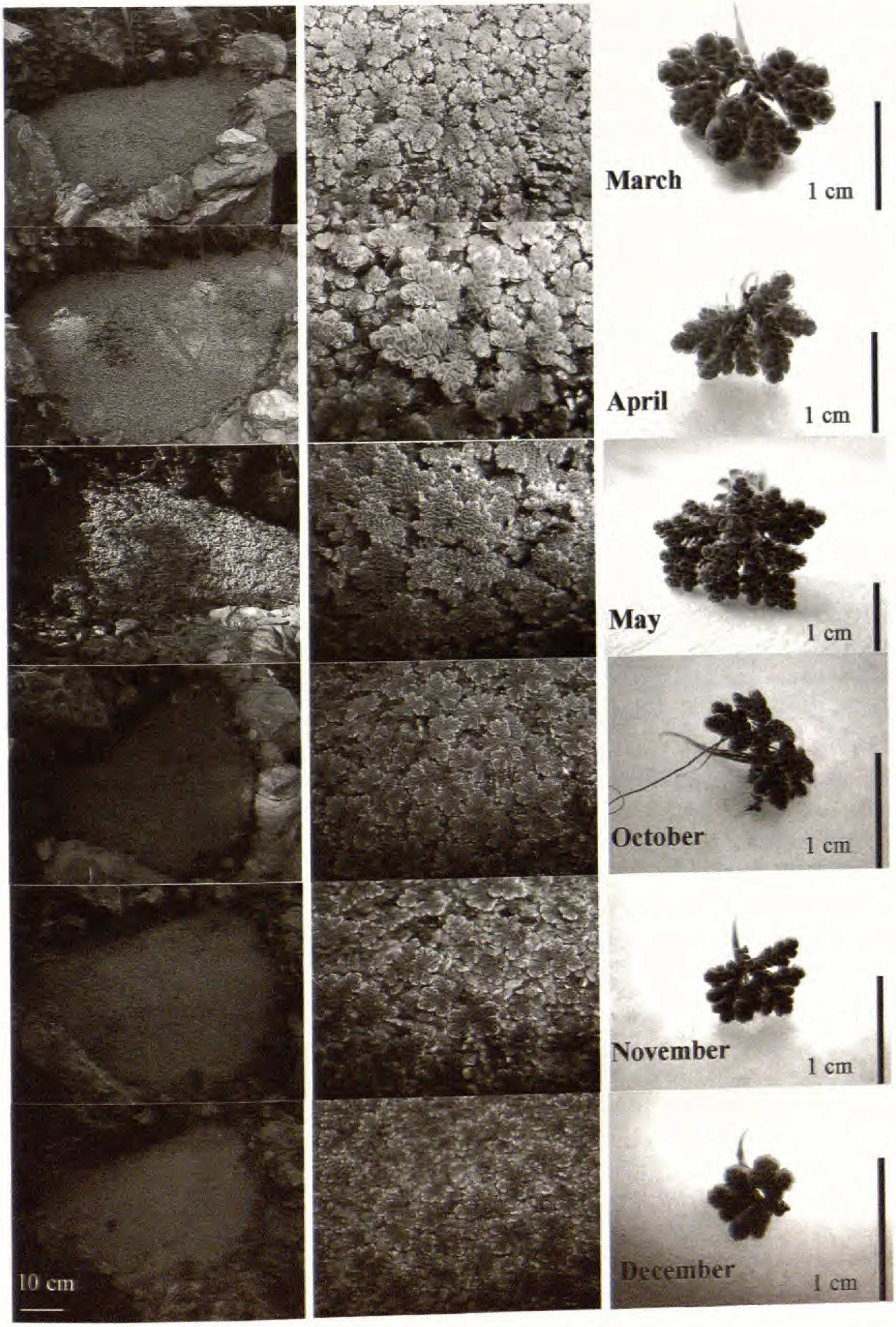

FIG. 2. Photographs of *A. filiculoides* in a small pond located in the Istanbul University Alfred Heilbronn Botany Garden in March, April, May, October, November, December 2007.

TABLE 1. Chlorophyll a, b (μg/gFW) and chl a and chl b ratio of *A. filiculoides* in March, April, May, October, November, December 2007. Chlorophyll a, b contents and chl a/chl b ratios results are means of 5 replicates in March-April-May and October-November-December, "±" indicates standard deviation. Significant differences determined by the Tukey's Multiple Comparison test ($p<0.05$) are indicated by different letters (a–e).

Months	Chlorophyll a	Chlorophyll b	Chl a/Chl b
March	197.2 ± 8.5 c	66.8 ± 2.0 a	2.9 ± 0.12 c
April	110.0 ± 12.2 e	47.1 ± 3.5 b	2.3 ± 0.14 e
May	170.6 ± 10.8 d	66.9 ± 1.4 a	2.6 ± 0.12 d
October	323.5 ± 23.8 a	63.4 ± 5.5 a	5.1 ± 0.14 b
November	269.1 ± 20.7 b	47.4 ± 4.1 b	5.7 ± 0.07 a
December	246.5 ± 10.4 b	43.7 ± 3.0 b	5.6 ± 0.15 a

In May, the plants had become fully green, were growing faster, and had completely covered the surface of the pond. During the October-November-December period, the color of the *Azolla* leaves changed from green to red, plant size decreased and plant growth also slowed down. In addition during the October-November-December period the *Lemna* spp. population density was higher than the *A. filiculoides* (Fig. 2). Although *A. filiculoides* was present in the small pond throughout the year 2007, it only reproduced vegetatively.

While the average chlorophyll a/b ratio of the leaves was 2.6 for the March-April-May period, it was 5.5 for the October-November-December period. In April the chlorophyll a/b ratio (Table 1) and total chlorophyll (Fig. 3) were significantly ($p<0.05$) lower than in the other months, while total chlorophyll was significantly highest in October. The total chlorophyll amounts were 264, 157, and 237 μg g^{-1} during March, April, and May, respectively (Fig. 3). The carotenoid amount of the *A. filiculoides* leaves was lower in the March-April-May period when compared to the October-November-December period. The highest carotenoid content was determined in October ($p<0.05$), but values for November and December were not statistically different from each other (Fig. 3).

While the highest level of anthocyanins was measured in March, the lowest was found in May, and these values are significantly different statistically from all other anthocyanin values measured. The anthocyanin content of the leaves was 0.6, 0.47, and 0.33 μg g^{-1}FW throughout October, November, and December respectively (Fig. 3).

DISCUSSION

Plants in their natural environment are exposed to different kinds of environmental stresses, and these conditions lead to both a reduction in growth and metabolism (Abraham, 2010). *Azolla* grows under different environmental conditions. Temperature is the most important factor affecting the growth and distribution of *Azolla* (Pabby *et al.*, 2004). The optimum growth

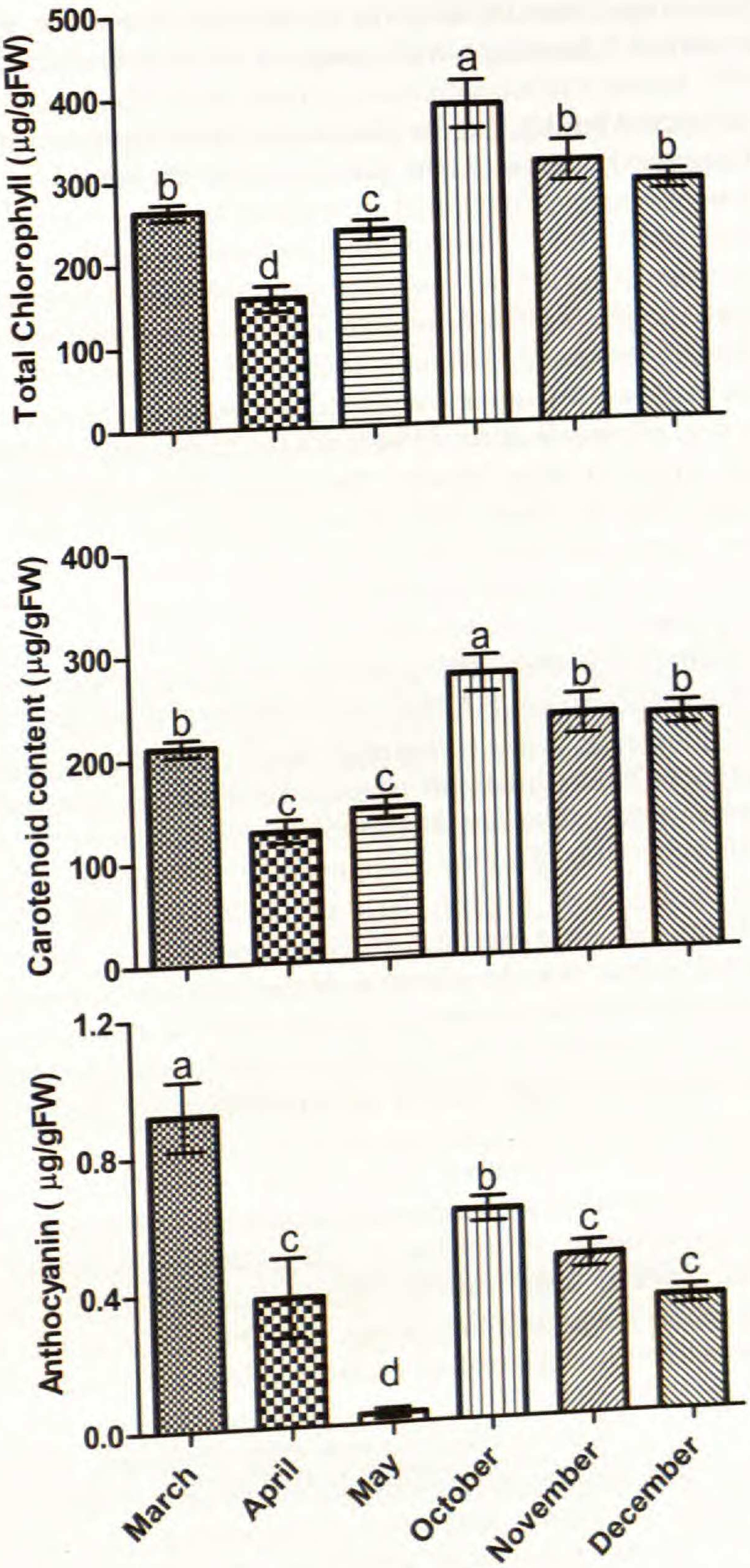

FIG. 3. Total chlorophyll, carotenoid and anthocyanin contents of *A. filiculoides* in March, April, May, October, November, December 2007. Bars represent the standard deviation. Significant differences determined by the Tukey's Multiple Comparison test ($p<0.05$) are indicated by different letters (a–d).

air temperature ranges from 18–28°C for *Azolla* spp. However, some species of *Azolla* are capable of tolerating a wide range of air temperatures from −5–35°C (Wagner, 1997). Janes (1998a), determined that under the most adverse winter conditions (January–March), *Azolla filiculoides* were very small and dark red, and in the spring/early summer, the plants' color changed from red to green. This color change was also observed in reverse in the autumn in an outdoor culture. In our study, the color and morphological results obtained from *A. filiculoides* in March-April-May, and October-November-December showed a parallelism with the Janes (1998a) results. Bocchi and Malgioglio (2010) stated that the *Azolla* (Milan strain) biomass increased from March to April (under the range from 10.4 to 18°C mean temperature/day). In the present study, the *A. filiculoides* leaf dimensions and the biomass increased and leaves returned to green from April to May (under the range from 12.4 to 19.9°C mean temperature/day; Fig. 2). Thus, the growth season of *A. filiculoides* likely began between April and May. This study also showed that under natural conditions, while *A. filiculoides* reproduced vegetatively, it did not reproduce sexually during 2007.

The photosynthetic apparatus is quite crucial for communication between the plant and the environment (Mattoo *et al.*, 1999). Chlorophyll concentration is regulated by plants in order to balance their capacity to both absorb and utilize light energy. This regulation is considered as an adaptation of plants to seasonal fluctuations under environmental stress (Close *et al.*, 2006). In May (providing the optimum growth temperature of plant), the amount of chlorophyll in leaves was higher than in April. On the other hand, the chlorophyll/carotenoid ratio was 1.6 in May and 1.2 in April. In addition, it was quite striking that the chl a/b ratio of the October-November-December months was almost twofold compared to the ratio in the months of March-April-May. Thus, in addition to its traditional role in energy transduction, the photosynthetic apparatus might also be an environmental sensor (Huner *et al.*, 1998).

With the synthesis of anthocyanins under high light intensity, low temperature, and in adverse environmental conditions, *Azolla* leaves generally have a reddish appearance. While the *A. filiculoides* population density was higher than the *Lemna* spp. during March-April-May, the opposite was observed in October-November-December. On the other hand, it was quite surprising that, while the amount of anthocyanins in March (mean temperature, 9.9°C) was 0.92 $\mu g g^{-1}$FW it was 0.33 $\mu g g^{-1}$FW in December (mean temperature, 7.6°C). Thus, anthocyanins may increase the antioxidant response of the plants against biotic and abiotic stress conditions.

Pigment synthesis in plants may be a consequence of exogenic stress or senescence, and of an ecological adaption to changing environments, respectively (Gould *et al.*, 1995). Thus, chlorophylls, carotenoids and flavonoids may contribute to maintaining a balanced physiological state in the plant tissues (Stintzing and Carle, 2004). With the amounts of chlorophyll, carotenoid and antocyanin evaluated together, the growth season of *A. filiculodies* likely began between April and May.

It is not yet known which environmental conditions induce *Azolla* to reproduce sexually. However, increased plant density, enhanced phosphate concentrations, and adverse winter conditions are factors believed to be involved in stimulating sexual reproduction in *Azolla* (Janes 1998a, 1998b). In conclusion, it is well established that changes in climate will affect the distribution of plant species (Malchair *et al.*, 2010; Rajkumar *et al.*, 2013) and agricultural productivity (Howden *et al.*, 2003). Thus the knowledge of the effect of seasonal changes on the photosynthetic pigment composition of *A. filiculoides* could help us in agriculture—especially in rice cultivation. In addition, it is important to investigate the effect of environmental conditions changing with global warming on the sexual reproduction and the formation of spores of *Azolla*, and encourage research regarding the preservation and germination of spores.

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